European Review for Medical and Pharmacological Sciences 2014; 18: 461-470

Protective effect of curcumin against contrast induced nephropathy in rat kidney: what is happening to oxidative stress, inflammation, autophagy and apoptosis?

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Abstract. – BACKGROUND: Currently, the number of imaging and interventional procedures that use contrast agents (CAs) is gradually increasing. Oxidative stress plays a significant role in its pathophysiology. Curcumin (CC) is a natural substance with strong antioxidant efficacy.

MATERIALS AND METHODS: In total, 24 male Wistar-albino rats were divided into four groups with seven rats in each group.

RESULTS: Biochemical measurements showed a significant increase ($p < 0.001$) in urea, creatinine and malondialdehyde (MDA) but a significant decrease ($p < 0.001$) in glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) levels in the contrast-induced nephropathy (CIN) group compared with the control group. The immunohistochemical examination revealed a significant increase in autophagic and apoptotic cell death ratios and in the inflammatory signal ($p < 0.05$). Compared with the CIN group, a significant improvement in these unfavorable parameters was observed with CC therapy.

CONCLUSIONS: The preventive efficacy of CC against an experimental model of CIN has been demonstrated.

Key Words:
Angiography, Nephropathy, Curcumin, Contrast, Autophagy, Apoptosis.

Introduction

Recently, the use of contrast agent (CA) has been gradually increasing simultaneously with an increase in imaging and interventional procedures. Despite advances in molecular structures, all CAs may have adverse effects, which range from mild to severe, with contrast-induced nephropathy (CIN) being the most important effect. CIN is the third leading cause of hospital-acquired acute renal failure[1]. Although the prevalence of CIN is 1-2% in the normal population, this prevalence increases by up to 50% in diabetic azotemic patients. The development of CIN prolongs the duration of hospital stays and enhances the requirement for dialysis and mortality[2].

Although the pathophysiology of CIN is unclear, at least four potential mechanisms have been suggested, including an alteration in renal perfusion, a direct tubular injury, oxidative stress and an immunological mechanism. Oxidative stress due to free radical production and direct tubular toxicity play basic roles in the development of CIN[3]. Nephropathy occurs due to necrotic and apoptotic cell death because of these pathophysiological mechanisms at the cellular level. Despite the use of various prophylactic therapies and low-osmolar CAs with lesser adverse effects, intravenous isotonic fluid infusion is the only method that has been proven effective in preventing CIN in clinic practice[4]. Therefore, a novel kidney-protecting treatment model is required for CIN.

Curcumin (CC) is the substance that gives yellow color to turmeric and that is responsible for its primary phytochemical effect. CC is a polyphenolic curcuminoid and accounts for 3-5% of turmeric. CC has strong antioxidant and anti-inflammatory effects. This substance shows antioxidant efficacy by inhibiting ROS production and scavenging molecular oxygen (O_2^-) and hydroxyl (OH^-) radicals[5]. Anti-inflammatory efficacy occurs by inhibiting LOX and COX pathways of arachidonic acid production. In previous studies, CC has been studied together with many nephrotoxic agents, and its pro-

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tective efficacy has been demonstrated[6]. A previous study investigated the protective efficacy of CC against CIN, and favorable outcomes were obtained over hemoxygenase-1 and over the apoptotic index[7]. Nevertheless, no large and comprehensive studies concerning the protective efficacy of CC in CIN have been conducted previously.

The present study investigated the effects of autophagy, apoptosis and inflammation that result from oxidative stress in CIN and the likely protective character of curcumin against these unfavorable effects.

Materials and Methods

Animals

In total, 24 adult male Wistar-albino rats, weighing 180-200 g were obtained from the Animal Laboratory at the Experimental Research Centre at Atatürk University, Erzurum, Turkey. The animals were maintained at standard housing facilities (24±1°C, 45±5% humidity and 12 h light/dark cycle). The animals were supplied with standard laboratory chow and water *ad libitum* and were left to acclimatize for one week before the experiments. The experimental protocol was approved by the Local Animal Care Committee at Atatürk University, Erzurum, Turkey, and experimental procedures were performed in accordance with the "International Guidelines for Care and Use of Laboratory Animals".

Drugs and Chemicals

Sevoflurane (Sevorane Liquid 100%) was obtained from Abbott Lab., Istanbul, Turkey. Furosemide (Desal amp.) was obtained from Biofarma, Istanbul, Turkey. Indometacin was obtained from Sigma Chemical Co., St Louis, MO, USA. Iomeprol (Iomeron flc. 400 mg/ml) was obtained from Bracco SpA, Milan, Italy. Curcumin was obtained from Sigma Chemical Company, USA.

Experimental Protocol

Rats were randomized into four groups with seven rats in each group. These groups were: healthy control rats (NC), rats that received curcumin (CC), rats that underwent contrast agent nephropathy (CIN) and rats that underwent contrast agent nephropathy and received curcumin (CIN+CC). The study period was 10 days. The application in each group was as follows:

Healthy control rats: 0.5 ml of corn oil was administered via gavage for 10 days. On the 5th day, i.m. 0.2 ml of saline + i.p. 0.2 ml of saline + IV 2 ml of saline was administered under mild sevoflurane anesthesia.

Rats that received curcumin: Curcumin was given at a dose of 200 mg/kg/day as a suspension in corn oil via gavage for 10 days.

Rats that underwent contrast agent nephropathy: 10 mg/kg furosemide IM + 10 mg/kg indomethacin IP + 10 ml/kg iomeprol IV were administered on the 5th day following 24-h dehydration under mild sevoflurane anesthesia[8].

Rats that underwent contrast agent nephropathy and received curcumin: Curcumin was administered at a dose of 200 mg/kg/day as a suspension in corn oil via gavage for 10 days. After 24-h dehydration on the 5th day, 10 mg/kg furosemide IM + 10 mg/kg indomethacin IP + 10 ml/kg iomeprol IV were administered under mild sevoflurane anesthesia.

Sample Preparation and Biochemical Studies

All animals in the groups were decapitated under mild sevoflurane anesthesia 24 h after the last application. Blood samples were collected into plain tubes and centrifuged at 200 x g for 5 min, and serums were extracted. One of the kidneys was removed for biochemical analysis, washed with normal saline and stored at −20 °C until the day of analysis. The other kidney was removed for histopathological analysis and storedin 10% buffered formaldehyde.

Serum urea and creatinine concentrations were measured using photometric commercial kits (Diasis Diagnostic Systems, Istanbul, Turkey).

The tissue homogenization was performed using a Teflon-glass homogenizer with a buffer that contained 1.15% KCl to obtain 1:10 (w/v) whole homogenate.

The renal tissue catalase (CAT) enzyme activity was determined by measuring the decomposition of hydrogen peroxide at 240 nm according to the method of Aebi and was expressed as katal/g protein[9]. In parallel, the protein concentration was also measured in the supernatants using the method of Lowry et al[10]. The tissue reduced glutathione (GSH) concentration was measured by a kinetic assay using a dithionitrobenzoic acid recycling method that was previously described by Ellman and was expressed as μmol/g protein[11]. The glutathione peroxidase (GSH-Px) enzyme activity was determined by the procedure that was previously described by Beutler[12]. The analysis procedure that was performed was based on the oxidation of GSH by GSH-Px, which was coupled to the disappearance of NADPH by glutathione reductase, measured at 37 °C and 340 nm and expressed as U/g

protein. Lipid peroxidation [as malondialdehyde (MDA)] levels in the renal homogenate were measured using the thiobarbituric-acid reaction according to the method of Placer et al[13]. The values of MDA were expressed as nmol/g tissue. The superoxide dismutase (SOD) enzyme activity determination was based on the production of H_2O_2 from xanthine by xanthine oxidase and on the reduction of nitroblue tetrazolium as previously described[14]. The product was evaluated spectrophotometrically at 560 nm. The results were expressed as U/g protein.

Histopathological examination of renal tissue

At the end of the experiment, the necropsy of the rats was performed, and kidney tissue samples were fixed in 10% neutral buffered formalin. Paraffin embedded blocks were routinely processed, and 5-µm thick sections were stained with hematoxylin-eosin and examined under a microscope and 10 randomly selected microscopic fields were examined under 20x magnification. The histopathological findings in the sections were graded as 0 (none), 1 (mild), 2 (moderate) or 3 (severe).

Immunohistochemical Examinations of Renal Tissue

To examine the protective effects of curcumin on apoptosis, autophagy and inflammation in the kidney, cleaved caspase-3, LC3/B and *i*NOS expression in the kidney were assessed by immunohistochemical staining. Kidney sections on polylysine-coated slides were fixed in 10% neutral buffered formalin, embedded in paraffin and were treated with cleaved caspase-3, LC3/B and *i*NOS antibodies for immunohistochemical analysis (Table I). The procedures were performed according to the manufacturer's recommended protocol for cleaved caspase-3, LC3/B and *i*NOS immunohistochemistry, with slight modifications. Negative controls included staining tissue sections with the omission of the primary antibody. The sections were graded as 0 (no staining), 1 (staining, 25%), 2 (staining between 25% and 50%), 3 (staining between 50% and 75%) or 4 (staining 75%).

Statistical Analysis

All analyses were performed using the program Statistical Package for Social Sciences version 17 (SPSS Inc, Chicago, IL, USA). The data are presented as the mean ± the standard error of means (SEM). A one-way analysis of variance (ANOVA) and post hoc Tukey's test were used to determine the differences between groups in terms of the studied parameters. A value of $p < 0.05$ was considered statistically significant.

Results

Effects of Curcumin on Serum Biochemical Analysis

There was no significant difference in serum urea and creatinine levels between NC and CC groups (Table II). CIN induction significantly ($p < 0.001$) increased both urea and creatinine levels compared with the NC and CC groups. Curcumin pre- and post-treatment resulted in a significant reduction in serum urea and creatinine levels (Table II).

Effects of Curcumin on Renal Biochemical Analysis

Kidney tissue SOD enzyme activity, CAT enzyme activity, GSH concentration and GSH-Px enzyme activity levels decreased significantly (Table II, $p < 0.001$) in the CIN group compared with the NC and CC groups (Table II). Kidney tissue MDA levels increased significantly ($p < 0.001$) in the CIN group compared with the NC and CC groups (Table II). Curcumin pre- and post-treatment resulted in a significant increase in tissue antioxidant profiles and in a significant reduction in tissue lipid peroxidation.

Effects of Curcumin on Renal Histopathology

Histopathological appearances in the experimental groups and in the control group are shown in Figure 1. It was observed that necrotic and de-

Table I. Antibody specificity, dilution rates and incubation times.

Antibody	Dilution rates	Incubation times	Commercially
Polyclonal rabbit active/Cleaved Caspase 3 antibody	1/200	10 minutes (RT)	Novus Biological (Cat No. NB600-1235)
Polyclonal rabbit LC3/B antibody	1/400	10 minutes (RT)	Abcam (Cat. No. ab15323)
Polyclonal rabbit iNOS antibody	1/400	10 minutes (RT)	Abcam (Cat. No. ab48394)

RT = room temperature.

Table II. Effect of curcumin (CC) on blood urea, creatinine, renal malondialdehyde (MDA), and glutathione (GSH) levels, as well as catalase, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities in rats that are exposed to contrast-induced nephropathy (CIN).

	NC	CC	CIN	CIN+CC
Serum urea (mg/dl)	63.3 ± 1.45	66.97 ± 1.70	90.10 ± 1.55[a]	72.56 ± 1.60[b]
Serum creatinine (mg/dl)	0.52 ± 0.01	0.57 ± 0.01	0.87 ± 0.01[a]	0.66 ± 0.01[b]
SOD(U/g protein)	1.73 ± 0.03	1.67 ± 0.02	1.11 ± 0.02[a]	1.40 ± 0.01[b]
Catalase (k/g tissue)	60.61 ± 0.88	59.46 ± 0.43	29.25 ± 0.88[a]	39.70 ± 0.61[b]
GSH (µmol/g tissue)	24.41 ± 0.46	25.43 ± 0.39	17.21 ± 0.31[a]	20.35 ± 0.44[b]
GSH-Px (U/g protein)	13.58 ± 0.21	13.80 ± 0.30	7.10 ± 0.17[a]	9.04 ± 0.21[b]
MDA (nmol/g tissue)	63.65 ± 0.95	61.84 ± 0.58	133.30 ± 1.36[a]	85.30 ± 1.60[b]

All of the values are expressed as the mean ± SEM, n = 7 in each group.
[a]$p < 0.05$ vs. the control group.
[b]$p < 0.05$ vs. the contrast-induced nephropathy group.

generative changes, as well as intertubular hemorrhage, were lower in the CIN+CC group compared with the CIN group (Table III, $p < 0.05$).

Effects of Curcumin on Renal Immunohistochemistry

*i*NOS expression in the control group and in the experimental groups is shown in Figure 2. Anti-*i*NOS-specific staining was observed predominantly in the tubular region and was rarely observed in the glomerular region in the CIN group. Immunopositivity was diffuse in the tubular epithelial cells in some regions, whereas immunopositivity was localized intensely on the luminal surface of tubular epithelial cells in other regions. It was observed that this specific staining was reduced in the CIN+CCgroup compared with the CIN group (Table IV, $p < 0.05$). Anti-*i*NOS-specific staining in the cytoplasm of the tubular epithelial cells displayed a diffuse spread.

LC3/B expression in the control and in the experimental groups is illustrated in Figure 3. Anti-

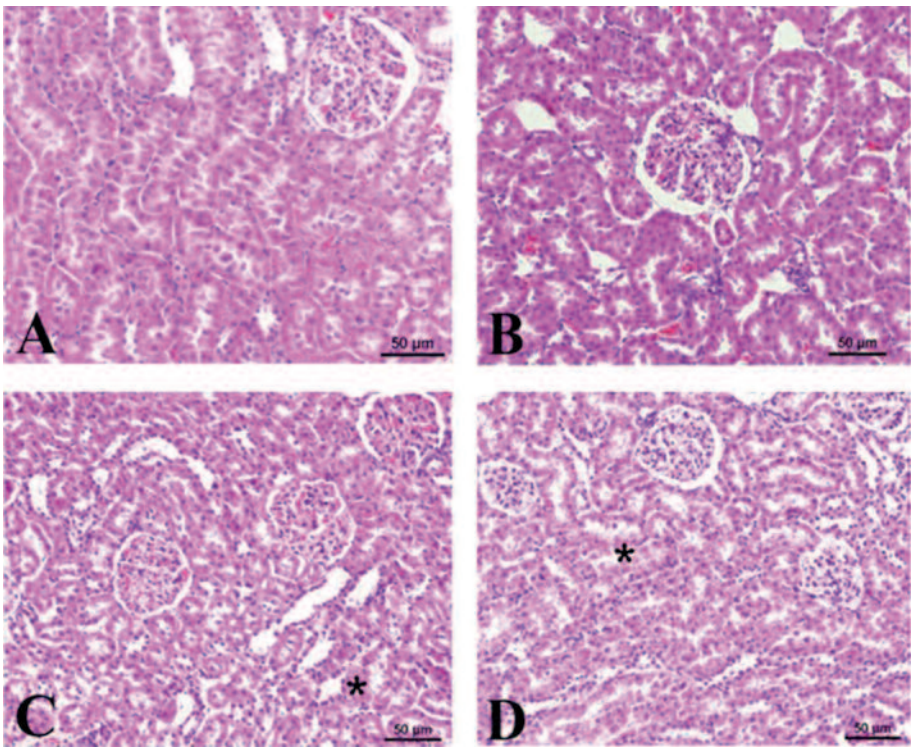

Figure 1. Photomicrographs of rat kidney (H&E, 200×) from: ***A,*** control group and ***B,*** curcumin alone-treated group showing normal renal architecture; ***C,*** curcumin with CIN group showing mild necrotic and degenerative tubular cells (*); ***D,*** alone CIN group showing moderate necrotic and degenerative tubular cells (*).

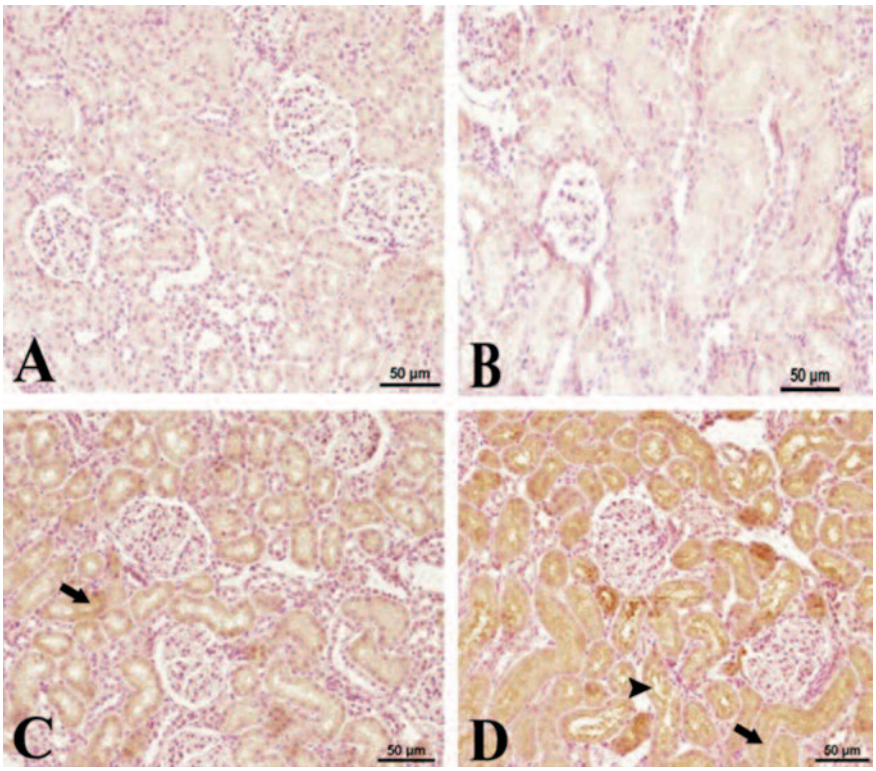

Figure 2. Immunohistochemical staining of *i*NOS in rat kidney (200×) from: ***A,*** control group and ***B,*** curcumin alone-treated group showing no expression of iNOS; ***C,*** curcumin with CIN group showing moderate immunopositivity in tubular cells *(arrow)*; ***D,*** alone CIN group showing severe immunopositivity diffusely *(arrow)* and in the luminal surface of tubular cells *(arrowhead)*.

LC3/B-specific staining was in the tubular epithelial cells, in podocytes and in mesangial cells in the glomeruli, as well as in cytoplasm and nucleus in the macula densa in the CIN group. Specific staining was observed to be reduced in the CIN+CC group compared with the CIN group (Table IV, $p < 0.05$).

Cleaved caspase 3 expression in the control and in the experimental groups is demonstrated in Figure 4. Anti-cleaved caspase 3-specific staining was predominant in the tubular region and was rarely observed in the glomerular regions in the CIN group. Immunopositivity was observed to be

Table III. Histopathological change rates in the groups.

	NC	CC	CIN	CIN+CC
Necrosis and degeneration	0.00 ± 0.00	0.00 ± 0.00	1.57 ± 0.20[a]	1.14 ± 0.14[b]
ntertubular hemorrhagia	0.00 ± 0.00	0.00 ± 0.00	1.42 ± 0.20[a]	1.00 ± 0.00[b]

NC = control; CC = curcumin; CIN = contrast-induced nephropathy
[a]$p < 0.05$ vs. the control group.
[b]$p < 0.05$ vs. the contrast-induced nephropathy + curcumin group.

Table IV. Immunohistochemical change rates in the groups.

	NC	CC	CIN	CIN+CC
iNOS	ND	ND	3.28 ± 0.18[a]	2.85 ± 0.14[b]
LC3/B	ND	ND	3.14 ± 0.14[a]	2.57 ± 0.20[b]
Active caspase 3	ND	ND	3.28 ± 0.80[a]	3.00 ± 0.21[b]

CC = curcumin; CIN = contrast-induced nephropathy; ND = non-detectable
[a]$p < 0.05$ vs. the control group.
[b]$p < 0.05$ vs. the contrast-induced nephropathy + curcumin group.

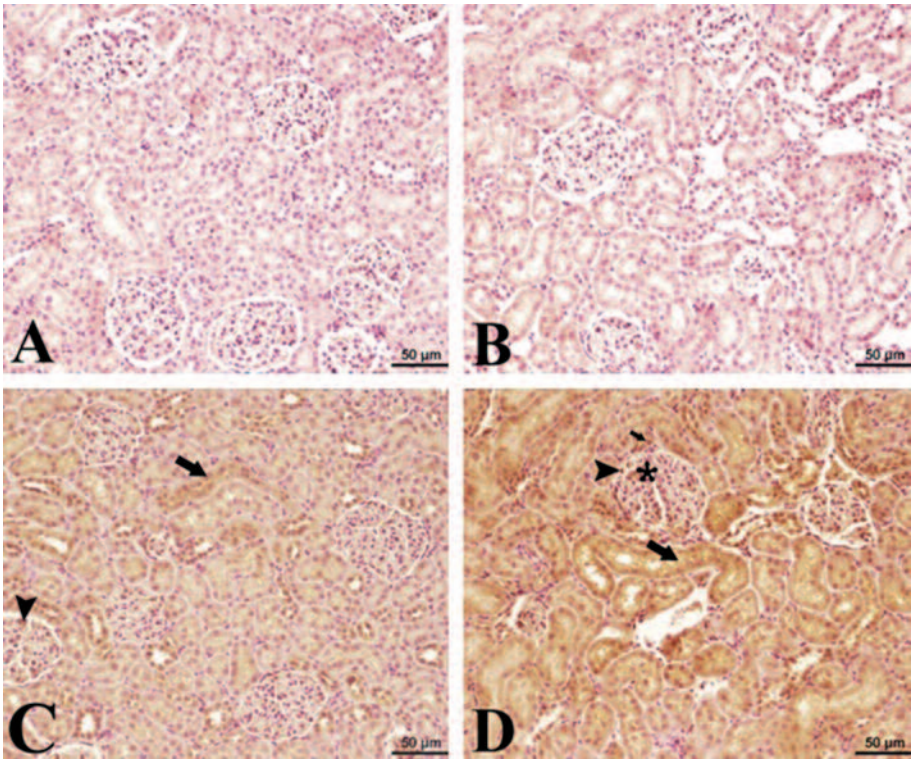

Figure 3. Immunohistochemical staining of LC3/B in rat kidney (200×) from: ***A,*** control group and ***B,*** curcumin alone-treated group showing no expression of LC3/B; ***C,*** curcumin with CIN group showing moderate immunopositivity in tubular cells *(arrow)*, podocytes (arrowhead); ***D,*** alone CIN group showing severe immunopositivity in tubular cells *(arrow)*, podocytes *(arrowhead)*, mesangial cell (*) and in the macula densa *(small arrow)*.

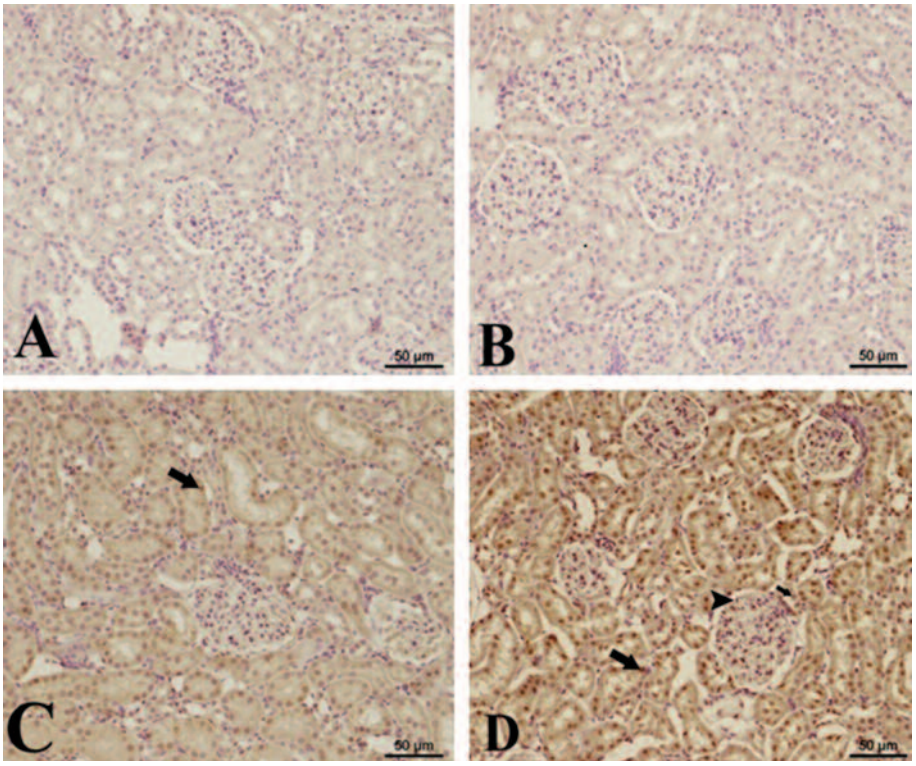

Figure 4. Immunohistochemical staining of cleaved caspase 3 in rat kidney (200×) from: ***A,*** control group and ***B,*** curcumin alone-treated group showing no expression of cleaved caspase 3; ***C,*** curcumin with CIN group showing moderate immunopositivity in tubular cells *(arrow)*; ***D,*** alone CIN group showing severe immunopositivity in tubular cells *(arrow)*, podocytes (arrowhead) and in the macula densa *(small arrow)*.

intensive in the nucleus but mild in the cytoplasm of the tubular epithelial cells. It was observed that this intense specific staining was reduced in the CIN+CC group compared with the CIN group (Table IV, $p < 0.05$). In this group, immunopositivity was detected in the tubular epithelial cells.

It was observed that apoptotic and autophagic cell deaths and the *i*NOS-induced inflammatory reaction, which were observed in the CIN group alone, were reduced in the CIN+CC group.

Discussion

The present study investigated biochemical, histopathological and immunohistochemical effects of CC against CIN.

Today, the prevalence of CIN increases as imaging and interventional procedures that are performed using CA increase and as these procedures are performed in older and comorbid patient groups. Although the pathophysiology of CIN has not yet been clarified, studies have brought oxidative stress into the forefront[15]. ROS production is increased because of adirect toxic effect of CA. Oxidative stress due to this increase in free radicals causes apoptosis in the renal tubular and glomerular cells[16]. Necrotic and degenerative changes that are observed particularly in tubular cells in CIN are consistent with the present study (Figure 1)[17].

Protective methods over these pathophysiological mechanisms have been explored in preclinical and clinical studies[8,18-20]. For example, an experimental study that was performed with melatonin demonstrated its protective and preventive effects[8]. In the experimental studies that were performed with alpha tocopherol and L-carnitine, the protective effects of these agents against CIN have also been demonstrated[19,20]. Most clinical studies examined saline, sodium bicarbonate, n-acetylcysteine (NAC), theophylline, dopamine, nitrendipine, furosemide, mannitol and ascorbic acid. Although the benefits of NAC have been demonstrated in previous studies, one of the recent studies found that NAC has no benefit in patients with at least one risk factor[21]. In the REMEDIAL trial, which was conducted in patients with renal failure, bicarbonate+NAC prevented CIN better than saline+NAC or saline+ascorbic acid[22]. Again, in a clinical study that was performed in patients with renal failure, ascorbic acid was shown to prevent CIN[23]. Finally, in 2008, there appeared results of a meta-analysis including 40 randomised controlled trials in which the following substances were administered: sodium bicarbonate, NAC, theophylline, dopamine, nitrendipine, statins, furosemide, mannitol, and ascorbic acid[24-29]. According to the results, only the administration of NAC or theophylline was more advantageous to patients than hydration with saline, while the use of furosemide significantly increased the risk of CIN[30-34]. In most of the studies, hydration with 0.9% NaCl resulted in a significant reduction in the risk of CIN. The effectiveness of patient hydration with the use of different methods is still ambiguous.

Curcumin is an herbal agent with antioxidant, anti-inflammatory and anticancer effects. These effects of CC have been demonstrated in many clinical studies. Among the studies that were performed with nephrotoxic agents, Waseem and Parvez[35] evaluated the mitochondrial LPO level, the protein carbonyl level and antioxidant enzyme parameters and demonstrated the protective effect of CC against cisplatin-induced nephropathy. Ueki et al[36] measured TNF-alpha and ICAM-1, which are among the inflammation parameters, and demonstrated the protective effect of CC against cisplatin-induced nephropathy. Kumaravel et al[37] indicate that the curcumin analog exhibit potent inhibitory activity in cancer cells. Sagiroglu et al[38] evaluated antioxidant enzymes and the apoptotic index and demonstrated the protective effect of CC against cyclosporine A-induced nephropathy. In the study that was conducted by Duan et al[7] on CIN, groups were compared in terms of hemoxygenase-1 activity and the apoptotic index, and the protective effect of CC against CIN was demonstrated. The latter study is the only one that was performed with CC in CIN.

Serum creatinine is the most important parameter that is used in the diagnosis of CIN in clinical practice. Previous studies determined a significant increase in both urea and creatinine in the CIN groups compared with the NC group[8,19,20]. In the present study, the serum creatinine level significantly increased after CIN induction in the CIN group compared with the NC group (Table II, $p < 0.001$). This result suggests that the protocol that we used has been successful in creating CIN. In the CIN+CC group, the urea and creatinine levels significantly decreased compared with the CIN group ($p < 0.001$).

MDA elevation in renal tissue is an indicator of an increase in lipid peroxidation due to nephrotoxicity. Previous studies of CIN and antioxidant agents demonstrated an increase in MDA levels in the renal tissue in CIN groups[19,20,39,40]. Again, in these studies, a significant decrease in MDA levels after treatment was demonstrated compared with the CIN group. In the present study, a significant increase was observed in MDA levels in the CIN

group compared with the NC group (Table II, $p < 0.001$). A significant decrease in the MDA level was observed with CC therapy compared with the CIN group ($p < 0.001$).

There are two types of antioxidant systems in the cell: enzymatic and non-enzymatic. In the present study, SOD, CAT, GSH-Px enzyme activities were measured as enzymatic antioxidants, whereas the GSH level was measured as a non-enzymatic antioxidant. Boyacioglu et al[20] found that SOD and CAT enzyme activities and the GSH level significantly decreased in the CIN group but significantly improved with L-carnitine therapy compared with the CIN group. Kongkham et al[19] determined that SOD enzyme activity decreased in the CIN group but significantly improved with alpha-tocopherol therapy. In the present study, it was observed that tissue SOD, CAT, GSH-Px enzyme activities and GSH levels significantly decreased in the CIN group compared with the control group (Table II, $p < 0.001$). A significant improvement was observed in these parameters with CC therapy compared with the CIN group (Table II, $p < 0.001$). This improvement in biochemical parameters with CC therapy can be explained by the antioxidant efficacy of CC.

In a study that was conducted in rats, it was observed that nitric oxide (NO) production was increased with CA[41]. It has been reported that particularly the *i*NOS form of NO, which has three different forms, such as endothelial nitric oxide synthase (*e*NOS), induced nitric oxide synthase (*i*NOS) and neuronal nitric oxide synthase (*n*NOS)[42], is produced in the presence of various cytokines and endotoxins, as well as in impaired cellular media[43]. It has been stated that *i*NOS can be found in extremely low amounts or not at all in normal renal tissue but increases in nephropathy[44]. In the present study, although *i*NOS expression was absent in the NC and CC groups, *i*NOS expression was the highest in the CIN group and was moderate in the CIN+CC groups (Figure 2, $p < 0.05$). This result indicates that CIN-induced *i*NOS expression was decreased by CC.

There are two different types of programmed cell death: apoptosis and autophagy[45]. Apoptosis is activated by apoptotic genes and by the caspase cycle. The activation of caspase-3 (cleaved caspase 3) plays an important role in the initiation and maintenance of apoptosis[46]. Some studies have demonstrated that CA has a cytotoxic or caspase-based apoptotic effect on renal tubular cells[47]. In the present study, the fact that cleaved caspase 3 is highly expressed in the CIN group but has decreased expression in the CIN+CC group suggests that the apoptotic effect of CA on renal cells might be alleviated with CC (Figure 4, $p < 0.05$).

Autophagy is a complex and well-organized hemostatic process that provides nutritional recycling by removing malfunctioned organelles and molecules to maintain life under stress. In contrast, changes in the rate of autophagy may lead to metabolic imbalance and cell death[48]. The appearance of the lipidated LC3 form among the membranes of autophagosomes is widely used as a marker of continuing autophagy[49]. In recent studies, it was stated that autophagy is present in nephrotoxin-associated acute renal injury and in nephropathic cystinosis[50,51]. In an experimental model of cisplatin-induced nephropathy, it was demonstrated that autophagy plays a role in cell death and that there is a precise balance between apoptosis and autophagy[52]. In the present study, LC3/B expression was the highest in the CIN group (Figure 3). Decreased expression of LC3/B in the CIN+CC group indicates that CC reduces autophagic cell death. Additionally, the fact that LC3/B expression is in line with cleaved caspase 3 indicates that both apoptosis and autophagy play a role in cell death.

Conclusions

We was observed that CC reduces inflammatory and programmed cell death in CA-induced renal injury; thus, CC might have protective efficacy against this condition.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Funding

This study was supported by the Scientific Research Fund of Erzincan University.

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